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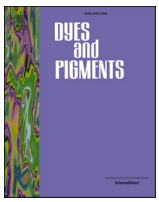

Biocatalytic synthesis of asymmetric water-soluble indirubin derivatives

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ABSTRACT

A method for the synthesis of asymmetric carboxy-substituted indirubins is presented. It employs indole-5-carboxylic acid or indole-6-carboxylic acid and 2-indolinone derivatives as substrates for bacterial monooxygenase-driven enzymatic bioconversion in different bacterial hosts. This bioconversion system achieved the highest titer of monocarboxyindirubin production of up to 327 mg L^{-1} for 5-bromoindirubin-6′-carboxylic acid during the 16-h incubation period. The purified monocarboxyindirubins exhibited high solubility in water, up to three orders of magnitude higher than that of indirubin. In addition, several monocarboxyindirubins, namely 1-methylindirubin-5′-carboxylic acid, possess potent antiproliferative activity against different cancer cell lines. Therefore, the synthesis method for monocarboxyindirubins described herein is an efficient and environmentally friendly bioconversion system and the synthesized monocarboxyindirubins show great promise due to their high water solubility and potential antiproliferative activity.

1. Introduction

Indigoids are a group of natural pigments composed of two oxygenated indole molecules, linked by a 2-2′ (indigo), 2′-3 (indirubin), or 3-3′ (isoindigo) double carbon-carbon bond (Fig. 1). A planar structure and a large conjugated system [1] are responsible for the spectral characteristics and deep colors, which are the hallmarks of indigoids. In addition, the presence of two carbonyl groups allows the reversible two-electron oxidation and reduction, giving indigoids a potential application as organic electron carriers [2,3].

Indirubin has a well-documented biological activity as an inhibitor of cyclin-dependent kinases 1 and 5 as well as glycogen synthase kinase [4]. Such activity suggests indirubin is a potent anticancer and antiproliferative agent. Indeed, several synthesized indirubin derivatives have shown potential activity against different types of cancer cells [5–7]. Indirubin derivatives can also be targeted to other targets inside the cancer cells or even have antibacterial properties [8]. Importantly, the antiproliferative extent of indirubin derivatives appears to depend on the solubility in aqueous solutions [7], but most synthetic indirubins are poorly soluble in water.

Chemical methods are available for the synthesis of substituted indirubins. The most common technique is a condensation of indoxyl and isatin derivatives to yield indirubin. Preparation of indoxyl usually results in a mixture of indigoid pigments. This can be avoided by using indoxyl acetate instead of indoxyl, which was demonstrated in the synthesis of derivatives of indirubin-5′-carboxylic acid [9,10] and indirubin-6′-carboxylic acid [10]. Alternatively, indirubin-5′-carboxylic acid and its derivatives can be synthesized from (2*Z*)-2-(1,2-dihydro-2-oxo-3H-indol-3-ylidene)-2-(phenylamino)ethanoic acids by a ring cyclization under acidic conditions and heating [11]. Furthermore, halogen moieties can be introduced into the carboxyindirubin backbone. Hence, 5-fluoroindirubin-5′-carboxylic acid was synthesized by the condensation of indoxyl acetate with 5-fluoroindoline-2,3-dione [12].

Several types of enzymes can catalyze the formation of indigoids by oxidizing indole. Such enzymes include flavoprotein monooxygenases, dioxygenases, Baeyer-Villiger monooxygenases, and cytochrome P450 monooxygenases [13]. The enzymatic oxidation of indole usually results in a combination of products: 3-hydroxyindole, 2-indolinone, and isatin which spontaneously dimerize to form a mixture of indigoids (indigo, indirubin, and isoindigo). Several methods and strategies have been proposed to direct the enzymatic synthesis towards indirubin and prevent the formation of other indigoids. Some enzymes can preferentially hydroxylate the C-2 position of indole, resulting in the formation of oxindole (2-indolinone) or isatin (as a result of 3-hydroxyindole

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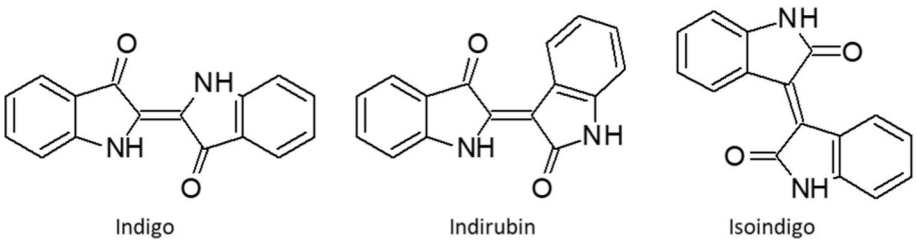


Fig. 1. Chemical structures of indigo, indirubin and isoindigo.

hydroxylation at C-2). In the presence of oxygen, oxindole or isatin then reacts with indoxyl to form indirubin. One example of such an indirubin-producing enzyme is cytochrome P450 BM-3. While the wild-type enzyme produces mainly indigo (>85%) from indole, a single D168W mutation shifts the production towards indirubin (>90%) [14]. Similarly, a bacterial flavin-containing monooxygenase from *Methylophaga aminosulfidovorans* has been modified by the structure-guided engineering strategy to achieve a high indirubin production reaching up to 113.4 mg L^{-1} [15].

Another approach to increase indirubin production is the addition of L-cysteine directly to the reaction mixture. Described a decade ago [16], this method was successfully used to produce high yields of indirubin with Baeyer-Villiger monooxygenase from *Acinetobacter radioresistens* (138 mg L^{-1}) [17] and flavin-containing monooxygenase (223.6 mg L^{-1} in a shaken flask bioconversion system [16] and 860.7 mg L^{-1} in a fed-batch fermentation [15]). Recently, the mode of action of L-cysteine addition has been elucidated [18]. First, L-cysteine reacts with indoxyl, thus preventing the dimerization of indoxyl into indigo. This complex can then react with 2-indolinone, a minor product of enzymatic indole oxidation, resulting in indirubin. At the same time, the L-cysteine promotes the enzyme-mediated hydroxylation of indoxyl at C-2, forming isatin. It is suggested that isatin can also react with the indoxyl-cysteine complex, forming indirubin. These findings have helped to understand how the addition of L-cysteine as a catalyst shifts the enzymatic oxidation of indole towards indirubin. It also shows that supplementing the enzymatic reactions with 2-indolinone or isatin can not only increase the production of indirubin, but also incorporate these additives into the final reaction product.

The final approach, which goes directly in line with the elucidated L-cysteine-mediated indirubin production mechanism, is to add 2-indolinone or isatin to the reaction. Unexpectedly, the addition of isatin only slightly improved the productivity of indirubin [19], and at high concentrations was thought to have inhibitory effects on pigment formation at high concentrations. On the other hand, 2-indolinone addition appears to be the most promising method to shift the oxidized indole dimerization towards indirubin. Using this approach, the yield of indirubin can be increased more than sixfold, reaching up to 60 mg L^{-1} in shaking flasks [19] and up to 250 mg L^{-1} in a continuous fermentation system [20]. Furthermore, the molar ratio of indigo: indirubin changed from 20:1 to 1:1 after the addition of 2-indolinone [19,20], indicating that this technique allows the production of high levels of indirubin with acceptable amounts of by-products. Finally, the fact that the added 2-indolinone becomes a part of the product molecule allows a simple method for the synthesis of asymmetric indirubin derivatives by using modified 2-indolinones [18].

Summing up the above, indirubin and its derivatives are potential anticancer molecules that can be synthesized by sustainable enzymatic methods and the activity of these compounds depends on their solubility in water. Thus, novel strategies for the synthesis of water-soluble indirubins could be valuable in the development of anticancer compounds. We aimed at the biocatalytic synthesis of a series of asymmetric water-soluble indirubin derivatives, taking advantage of two unique enzymes - the flavin-containing monooxygenase Hind8 [21] and a phenol monooxygenase-type system PmlABCDEF [22], both of which can oxidize indole carboxylic acids into the corresponding water-soluble indigo dicarboxylic acids.

2. Materials and methods

2.1. Chemicals, bacterial strains, and plasmids

Indole, 2-indolinone, 1-methyl-2-indolinone, 5-chloro-2-indolinone, 6-chloro-2-indolinone, 7-chloro-2-indolinone, 5-bromo-2-indolinone, 6-bromo-2-indolinone, 5-nitro-2-indolinone, 5-fluoro-2-indolinone, 5-amino-2-indolinone, L-arabinose, and gentamycin were purchased from Sigma-Aldrich (Munich, Germany), isopropanol – from Fisher Scientific (Loughborough, UK), kanamycin – from Carl Roth GmbH (Karlsruhe, Germany).

E. coli strain DH5α was used for cloning and plasmid isolation. *E. coli* strain HMS174 Δ*tnaA* [21] and *Pseudomonas putida* KT2440 (DSM 6125) were used as bioconversion hosts for the synthesis of asymmetric indirubins.

Plasmid pBAD2-MCS-1 (Km^r) [23] was used to clone the Hind8 oxygenase-encoding gene. pJNT plasmid (Gm^r) (a gift from prof. Dirk Tischler) was used for the cloning of PML G109Q (G109Q mutation in the catalytic subunit PmlD) oxygenase-encoding gene.

2.2. Cloning of Hind8 and PML G109Q genes into Pseudomonas expression plasmids

Hind8-encoding gene was cloned by excising the XbaI/HindIII fragment from pLATE31-Hind8 plasmid [21] and ligating it into the pBAD2-MCS-1 plasmid pre-digested with the same restriction endonucleases to create pBAD2-Hind8. The gene encoding PML G109Q was excised from the plasmid pET28-G109Q [21] with XbaI/NotI restriction endonucleases and ligated into the XbaI/NotI site of the plasmid pJNT [24], yielding the plasmid pJNT-G109Q. *E. coli* strain DH5α was transformed with the ligation mixtures by electroporation and the resulting suspension was plated on LB plates containing the appropriate antibiotic. Positive clones were identified by plasmid isolation and sequencing. All cloning reagents (XbaI, HindIII, NotI, T4 DNA ligase, Plasmid miniprep kit) were purchased from Thermo Fisher Scientific Baltics (Vilnius, Lithuania) and used according to the manufacturer's recommendations.

2.3. Whole-cell synthesis of asymmetric indirubins

Different conditions (**A**, **B,** and **C**) were tested for the synthesis of indirubins with Hind8 and PML G109Q enzymes. **Conditions A**: synthesis in *Escherichia coli* HMS174 Δ*tnaA* cells was performed exactly as described in [21] except that two substrates (2-indolinone or its derivatives and either indole-5-carboxylic acid or indole-6-carboxylic acid) were added to the bioconversion mixture. Different molar ratios of the two substrates were tested – 1:1 and 1:2 (indole carboxylic acid:2-indolinone derivative), keeping the concentration of indole carboxylic acid constant at 1 mM. Cell growth and protein synthesis were performed in 1 L flasks containing 200 mL of the appropriate medium. Bioconversion reactions were carried out in 100 mL flasks containing no

more than 20 mL of the bioconversion mixture. **Conditions B**: for synthesis in *Pseudomonas putida* KT2440, cells carrying either pBAD2-Hind8 or JNT-G109Q plasmid were grown overnight in LB medium supplemented with 40 μg mL $^{-1}$ kanamycin or 20 μg mL $^{-1}$ gentamycin, respectively. An overnight culture was diluted 100-fold in a fresh LB medium supplemented with the appropriate antibiotic. Cells were incubated with agitation (200 rpm) at 30 °C until reaching OD_{600} 0.4–0.5. Protein synthesis was induced by adding L-arabinose (final concentration 0.2%) for pBAD2-Hind8 or sodium salicylate (final concentration 1 mM) for pJNT-G109Q. Cells were further incubated at the same conditions overnight (>16 h). After the incubation, cells were pelleted by centrifugation at 3200×*g* for 10 min, washed with buffer A, and re-suspended in buffer A to reach the final concentration 5-fold higher than the concentration of the overnight culture. Indole-5-carboxylic acid (for pJNT-G109Q) and indole-6-carboxylic acid (for pBAD2-Hind8) were added to a final concentration of 2 mM together with 2-indolinone or its derivative (final concentration 2 or 4 mM) to initiate the bioconversion. Whole-cell bioconversion was performed by incubating the mixture at 30 °C with agitation (200 rpm) for 16–40 h until a deep blue-purple color was visible. **Conditions C:** the growth of *P. putida* KT2440 cells with pBAD2-Hind8 or pJNT-G109Q, protein synthesis, and whole-cell bioconversion was performed according to [25], but with indole carboxylic acids and 2-indolinone derivatives as described above. Under all tested conditions, different 2-indolinone: indole carboxylic acid molar ratios were examined (1:1, 2:1) to increase the yield of the indirubin form. All bioconversion reactions were carried out for 16 h.

The appropriate dicarboxylic acid and indirubin derivative were the only observable products under the given reaction conditions. Hence, the concentration of monocarboxyindirubin ($C_{indirubin}$) derivatives was calculated as follows: $C_{indirubin} = C_{initial} - (C_{subst} + 2 \times C_{indigo})$, where $C_{initial}$ – concentration of indole carboxylic acid before the conversion (was set constant at 1 mM), C_{subst} – concentration of indole carboxylic acid after the bioconversion, C_{indigo} – concentration of indigo dicarboxylic acid after the bioconversion. The concentration of indole carboxylic acids and indigo dicarboxylic acids was determined by using standard sample concentrations of these compounds and integrating the peak area from the HPLC chromatograms. Reaction yield (expressed in percentage) was defined as a ratio between the amount of obtained monocarboxyindirubin after purification and a theoretical maximum yield. The reaction titer was calculated as follows: purified monocarboxyindirubin (mg)/total bioconversion reaction volume (L). The total bioconversion reaction volumes were in the range of 0.1–0.8 L.

2.4. Purification of indirubins

After bioconversion, the medium was supplemented with isopropanol to a final concentration of 30% and mixed well to facilitate the recovery and extraction of indigoids. Bacterial cells were pelleted by centrifugation at 7197×*g* for 15 min. The supernatant was diluted tenfold to a final concentration of 3% isopropanol and loaded onto C18 Reveleris® Flash column (Grace), pre-equilibrated with 3 column volumes of buffer A. Target compounds were eluted with a linear gradient of isopropanol (0–30%) and colored fractions were analyzed for purity by HPLC/MS. Purified compounds were dried *in vacuo* and subjected to further analyses.

2.5. Characterization of the purified indirubins

^{1}H and ^{13}C NMR spectra of reaction substrates and products were recorded in DMSO-D_6 on a Bruker Ascend 400 at 400 MHz (^{1}H) and 101 MHz (^{13}C). Spectra were calibrated with respect to the solvent signal (DMSO-D_6, ^{1}H δ = 2.50). NMR spectra of 2-indolinones and the corresponding monocarboxyindirubins are provided as Supplementary Material.

High-resolution mass spectrometry (HRMS) mass spectra of the synthesized compounds were recorded by Agilent TOF 6230 equipped with Agilent Infinity 1260 HPLC, in negative electrospray ionization (ESI) mode. Isocratic elution of 80% acetonitrile with 20% deionized (18.2 MΩ cm @ 25 °C) water was used at a flow rate of 0.300 mL min^{-1}.

The water solubility of purified indirubins was determined as described earlier [21].

2.6. Cell lines

All procedures were carried out under aseptic conditions meeting biological safety requirements. HCT116 cells (a gift from dr. Daiva Baltriukienė) are human colon cancer cells derived from the colon of an adult male, a colon cancer patient. MCF7 cells (also a gift from dr. Daiva Baltriukienė) are human epithelial cells isolated from the breast tissue of an adult female, patient with metastatic adenocarcinoma. Both cell lines were maintained in DMEM (Gibco) containing 10% fetal bovine serum (FBS, Gibco), 100 U ml^{-1} penicillin, and 0.1 mg mL^{-1} streptomycin (Gibco). Cells were grown at 37 °C with 5% CO_2 in a water-saturated incubator. For passaging, cells were incubated with trypsin/EDTA (Corning) at 37 °C to detach cells.

2.7. MTT assay

HCT116 and MCF7 cells were cultured on 96-well plates at a density of 4000 cells/well. The cells were incubated for 24 h to allow cells to attach to the culture vessel before being exposed to several compound concentrations for 24–48 h. After that, the culture medium was carefully removed and 100 μl of the MTT solution (0.5 μg mL^{-1} MTT reagent (Merck) in PBS) was added to each well. The metabolically active cells reduced MTT to blue formazan crystals. After 1 h of incubation at 37 °C, MTT-formazan crystals were dissolved in 120 μl DMSO, 100 μl of which was transferred to a new 96-well plate suitable for optical measurements. The absorbance of the colored formazan product was measured at 540 and 650 nm by Varioskan Flash Spectrophotometer (ThermoFisher Scientific). The untreated cells served as a negative control group. Prior to performing statistical analysis, the obtained data were processed in three steps: first, the 650 nm measurements (background) were subtracted from each 540 nm measurement; the mean measurement value obtained from the samples treated with DMSO was subtracted from the control group and each test cell sample group treated with different compounds; viability of the cells was evaluated by comparison of test samples against the negative control and expressed as percentage considering that viability of untreated cells was 100%. Each test group was tested in eight replicates.

Solutions of all compounds were prepared fresh for each experiment in DMSO diluted in the complete culture medium and added to HCT116 and MCF7 cells to a final concentration of 10 and 100 μM. The concentration of DMSO in the assay never exceeded 0.02% and did not influence cell growth.

2.8. Statistical analysis

All grouped MTT analysis data were presented as mean with a 95% confidence interval. Comparisons between groups were made by the one-tailed Welch's *t*-test for independent samples. The Shapiro-Wilk test [26] was performed to evaluate deviation from normality. The significance level was chosen at $\alpha = 0.05$ for all criteria used. Data were plotted and statistical analysis was performed using RStudio version 1.3.1073 [27].

For bioconversion, two independent bioconversion reactions were performed with each substrate, and mean values are presented. The standard deviation was less than 15% in all experiments.

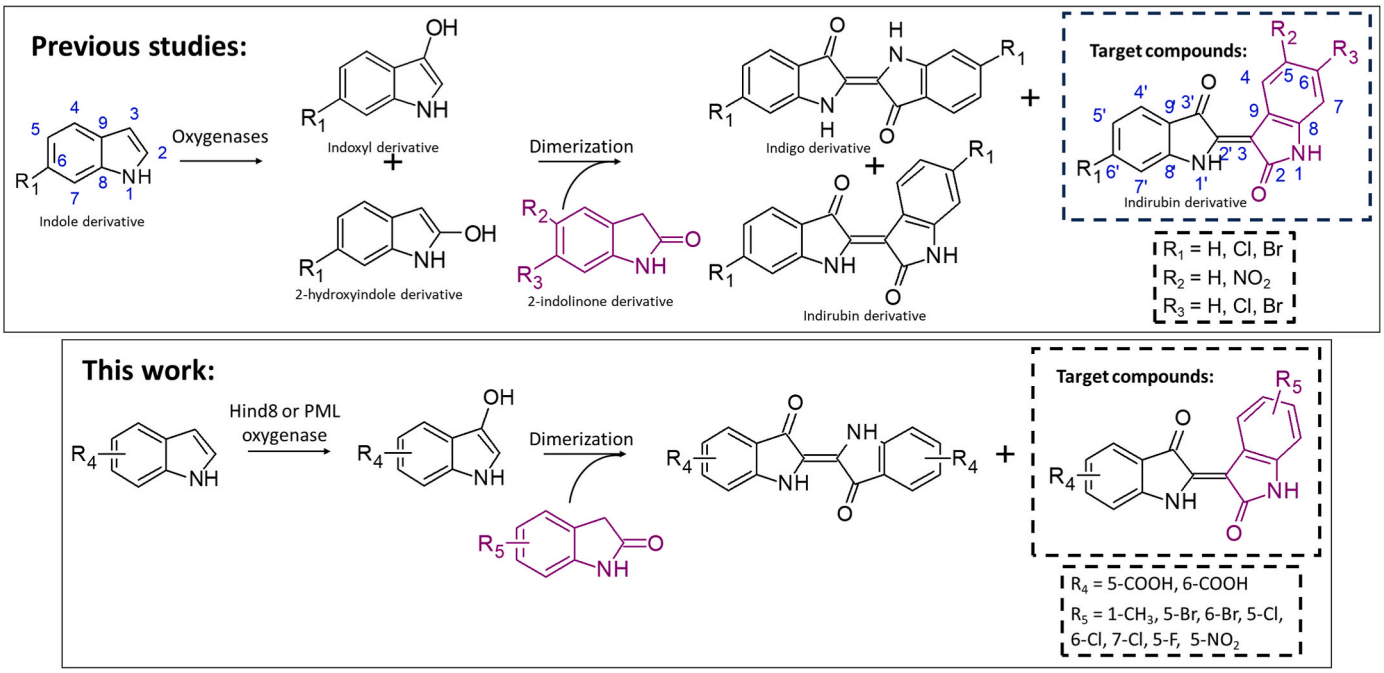


Fig. 2. The proposed conversion pathway of indole into indigo and indirubin by adding 2-indolinone [18,20] and production of asymmetric water-soluble monocarboxyindirubins by adding modified 2-indolinones. Specific oxidation of indole carboxylic acids towards indoxyl carboxylic acids by Hind8 and PML oxygenases is inferred from the absence of reaction products other than indigo [21,25].

3. Results

3.1. Synthesis of monocarboxyindirubins

Enzyme-based production of modified indirubins can be a valuable alternative to chemical synthesis [13,21,28]. One such method used indole carboxylic acids as substrates for two specific monooxygenase systems: a group B flavin-dependent monooxygenase Hind8 and a PmlD G109Q mutant of the phenol monooxygenase-like protein (PML). The resulting products were identified as indigo and indirubin dicarboxylic acids, which showed relatively high solubility in aqueous solutions. Recently, other research groups showcased a technique for directing the enzymatic oxidation of indole to produce indirubin and its asymmetric derivatives (Fig. 2). While such a reaction usually results in the mixture of indigo and indirubin (and, occasionally, isoindigo), the addition of 2-indolinone (oxindole) to the indole oxidation reaction, catalyzed by a novel terpenoid cyclase XiaI, triggered an almost 100-fold increase in indirubin production [20]. In this work, we first tested the possibility of combining these two methods for the production of asymmetric monocarboxyindirubins. For this, we employed tryptophanase-negative *E. coli* HMS174 Δ*tnaA* bacterial cells carrying either Hind8 or PML G109Q enzymes and two different substrates – indole carboxylic acid and 2-indolinone – in a single whole-cell bioconversion reaction. Due to their different substrate preferences [21], indole-5-carboxylic acid was provided for the PML G109Q enzyme and indole-6-carboxylic acid was provided for the Hind8 enzyme. Since the oxidation of indole-5-carboxylic acid and indole-6-carboxylic acid by PML G109Q and Hind8 monooxygenases, respectively, yields a single indigo dicarboxylic acid, the addition of 2-indolinone could theoretically produce a mixture of two products: indigo dicarboxylic acid and indirubin monocarboxylic acid (Fig. 2). This was indeed the result that we observed. Bioconversion of indole-5-carboxylic acid and 2-indolinone with *E. coli* HMS174 Δ*tnaA* cells carrying the PML G109Q enzyme resulted in two products (Fig. S2): indigo-5,5′-dicarboxylic acid (retention time 5.8 min [21]) and a new product (retention time 6.25 min) with a molecular weight of 306 Da and an absorbance peak at 542 nm (Fig. S22). Similarly, when using Hind8-containing *E. coli* HMS174 Δ*tnaA* cells for bioconversion and indole-6-carboxylic acid with 2-indolinone as substrates, two products were obtained (Fig. S3): indigo-6,6′-dicarboxylic acid (retention time 6.0 min [21]) and a new product (retention time 6.8 min) with molecular weight 306 Da and absorbance peak at 557 nm (Fig. S22). These new products were later purified and their structures were confirmed by NMR as indirubin-5′-carboxylic acid and indirubin-6′-carboxylic acid, respectively.

Next, we hypothesized that by introducing modified 2-indolinones instead of 2-indolinone, the appropriately modified indirubins could be obtained in this bioconversion system. To test this, nine different 2-indolinones (1-methyl-2-indolinone, 5-chloro-2-indolinone, 6-chloro-2-indolinone, 7-chloro-2-indolinone, 5-bromo-2-indolinone, 6-bromo-2-indolinone, 5-fluoro-2-indolinone, 5-nitro-2-indolinone, and 5-amino-2-indolinone) were used in bioconversion reactions together with either indole-5-carboxylic acid or indole-6-carboxylic acid. The corresponding asymmetric monocarboxyindirubin derivatives were observed in most of these reactions (Figs. S4–S19), except for 5-amino-2-indolinone, where several unidentified products were obtained (Figs. S20 and S21). This could be explained by the intrinsic activity of *E. coli* towards the amino group of 5-amino-2-indolinone or by the reactivity and instability of the substrate itself.

3.2. Optimization of bioconversion conditions

Although our bioconversion system worked with a variety of substrates, the yield of indigoids and indirubins in particular was insufficient under the conditions tested. (Fig. 3A). To further characterize the newly synthesized compounds and to prove their structure, different reaction conditions were tested for the production of indigoids in order to increase the yield of indirubin derivatives. Based on previous observations [29], *P. putida* KT2440 was chosen as the bioconversion host, along with the *E. coli* HMS174 Δ*tnaA* system. Importantly, no intrinsic activity of *P. putida* KT2440 was found towards any of the tested 2-indolinone derivatives or indole-5-carboxylic acid and indole-6-carboxylic acid. In addition, *P. putida* KT2440 does not produce indole [30], eliminating unwanted intrinsic oxidation of indole to indigo by Hind8 and PML G109Q.

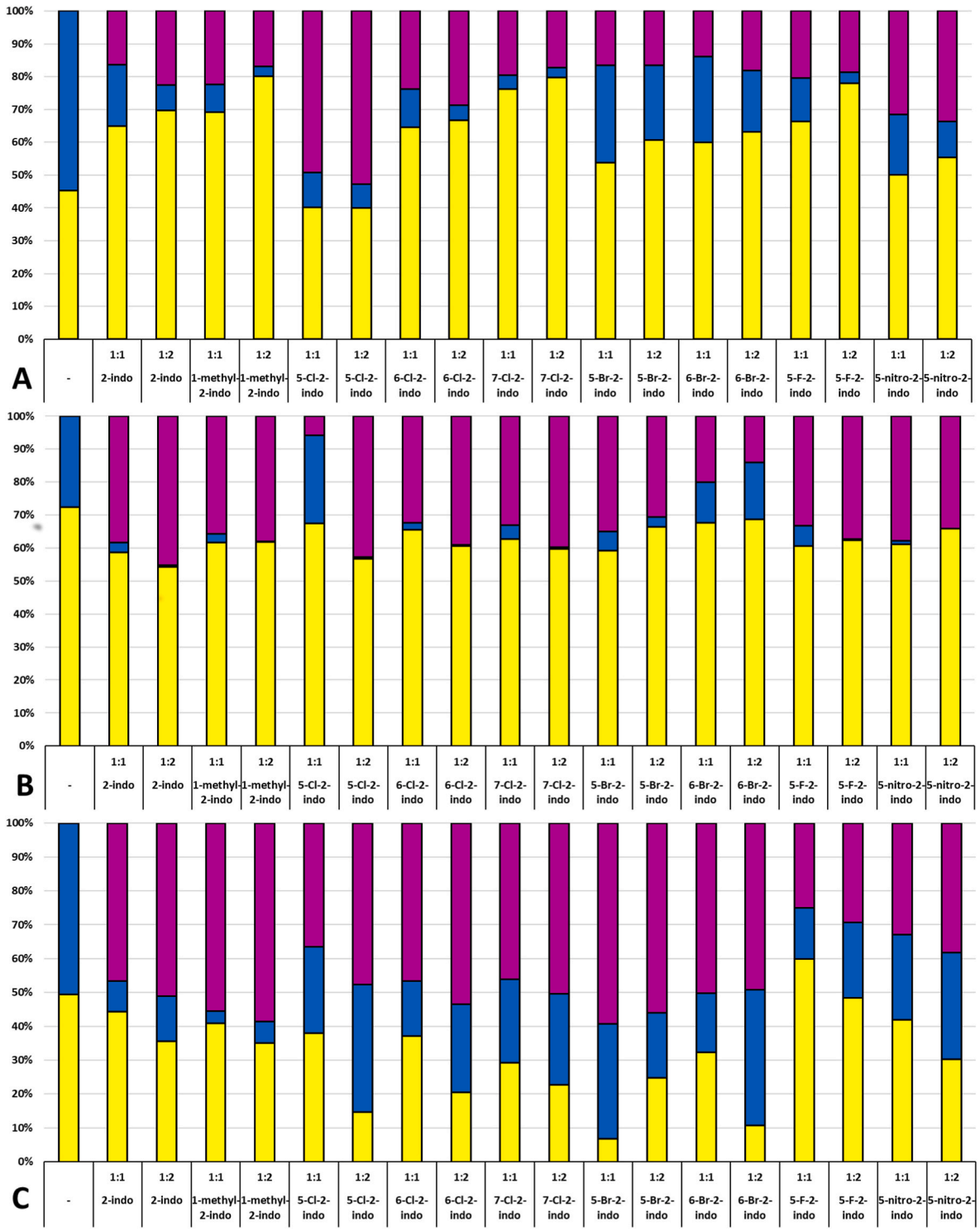


Fig. 3. Optimization of the synthesis of indirubin-5′-carboxylic acid and its derivatives with PML G109Q monooxygenase. Reaction products and residual substrate amounts were analyzed in the bioconversion mixture. A – synthesis in *E. coli* Δ*tnaA,* B – synthesis in *P. putida* KT2440 under regular conditions, C – synthesis in *P. putida* under optimized conditions. Yellow – residual indole-5-carboxylic acid, blue – indigo dicarboxylic acid formed during the bioconversion, purple –the corresponding monocarboxyindirubin derivative formed during the bioconversion. Here, 100% of 2-indolinone and monocarboxyindirubin derivatives is equivalent to 1 mM, 100% of indigo dicarboxylic acid −0.5 mM. The following abbreviation were used: 2-indo – 2-indolinone, 1-methyl-2-indo – 1-methyl-2-indolinone, 5-Cl-2-indo – 5-chloro-2-indolinone, 6-Cl-2-indo – 6-chloro-2-indolinone, 7-Cl-2-indo – 7-chloro-2-indolinone, 5-Br-2-indo – 5-bromo-2-indolinone, 6-Br-2-indo – 6-bromo-2-indolinone, 5-F-2-indo – 5-fluoro-2-indolinone, 5-nitro-2-indo – 5-nitro-2-indolinone.

The synthesis of indirubin carboxylic acid derivatives was tested under three different conditions: (i) regular conditions in *E. coli* HMS174 Δ*tnaA*, (ii) regular conditions in *P. putida* KT2440, and (iii) optimized conditions in *P. putida* KT2440. Detailed protocols for these conditions are provided in the Materials and Methods section. The efficiency of these syntheses was evaluated by comparing the production of the target compound (a derivative of indirubin carboxylic acid) and the amount of the side-product (indigo dicarboxylic acid). The synthesis of indirubin-

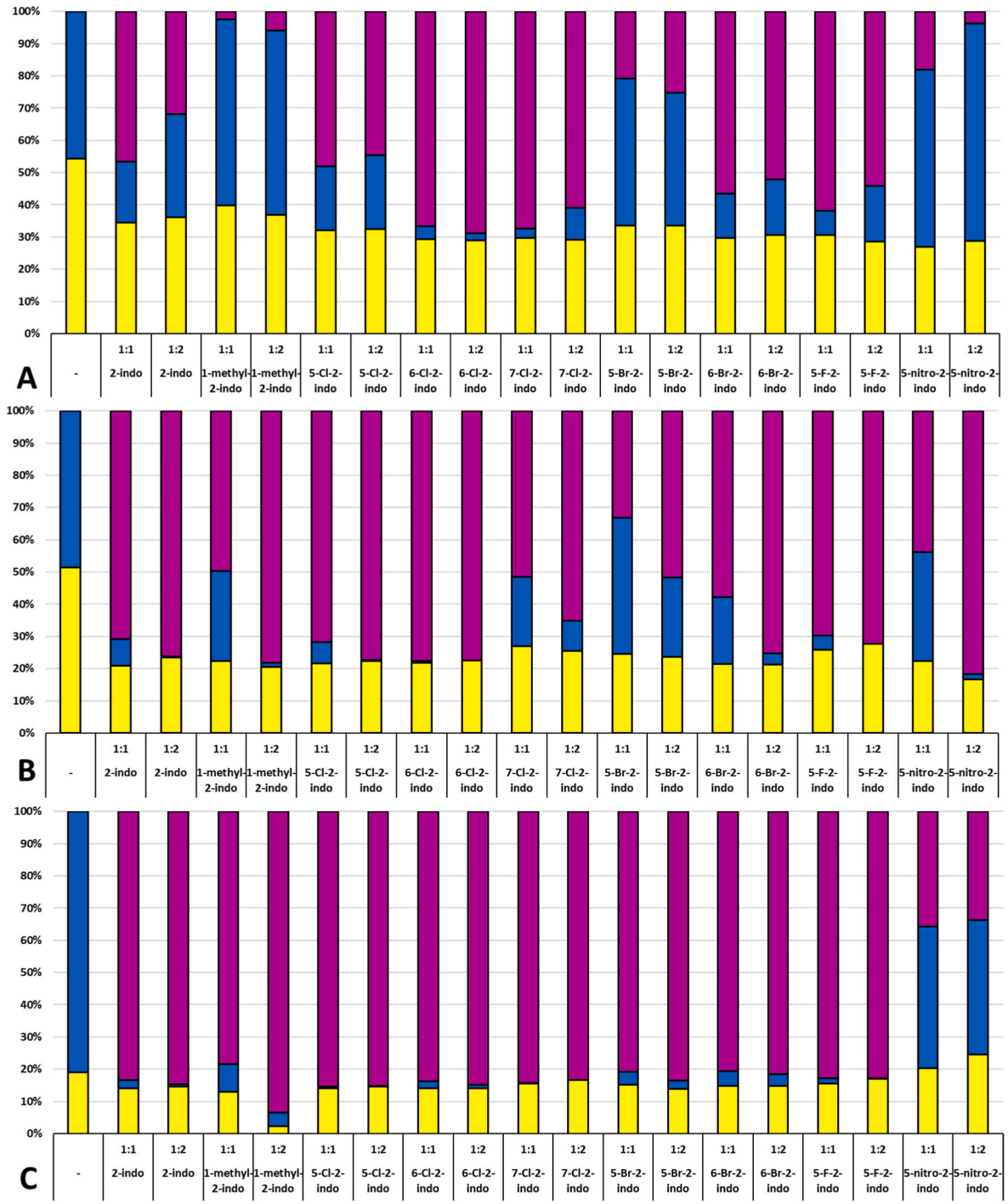
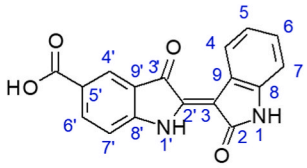
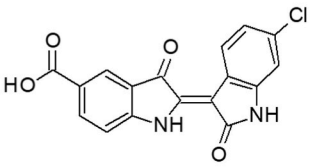


Fig. 4. Optimization of the synthesis of indirubin-6-carboxylic acid and its derivatives with Hind8 monooxygenase. Reaction products and residual substrate amounts were analyzed in the bioconversion mixture. A – synthesis in *E. coli* Δ*tnaA,* B – synthesis in *P. putida,* C – synthesis in *P. putida* under optimized conditions. Yellow – residual indole-6-carboxylic acid, blue – indigo dicarboxylic acid formed during the bioconversion, purple –the corresponding monocarboxyindirubin derivative formed during the bioconversion. Here, 100% of 2-indolinone and monocarboxyindirubin derivatives is equivalent to 2 mM, 100% of indigo dicarboxylic acid – 1 mM The following abbreviations were used: 2-indo – 2-indolinone, 1-methyl-2-indo – 1-methyl-2-indolinone, 5-Cl-2-indo – 5-chloro-2-indolinone, 6-Cl-2-indo – 6-chloro-2-indolinone, 7-Cl-2-indo – 7-chloro-2-indolinone, 5-Br-2-indo – 5-bromo-2-indolinone, 6-Br-2-indo – 6-bromo-2-indolinone, 5-F-2-indo – 5-fluoro-2-indolinone, 5-nitro-2-indo – 5-nitro-2-indolinone.

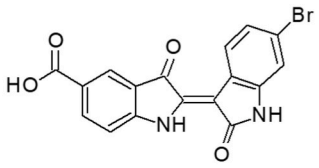
5′-carboxylic acid by PML G109Q in *P. putida* KT2440 under regular conditions resulted in a molar ratio of indirubin-5-carboxylic acid: indigo-5,5′-dicarboxylic acid of approximately 10:1, compared to the ratio of 3:1 in *E. coli* HMS174 Δ*tnaA* (Fig. 3B). The production yield of indirubin-5′-carboxylic acid was also increased 2-fold. Under optimized bioconversion conditions, *P. putida* KT2440 further increased titer to 162 mg L^{-1}. A similar pattern was observed with other derivatives of 2-indolinone. The efficiency of bioconversion was much higher under the optimized conditions as indicated by the lower levels of residual indole-5-carboxylic acid (Fig. 3C). In most cases, this resulted in a lower indirubin-5′-carboxylic acid: indigo-5,5′-dicarboxylic acid ratio, but the overall productivity of the indirubin derivative was still higher compared to the productivity in *E. coli.* The notable exception was 5-chloroindirubin-5′-carboxylic acid, which was produced in higher



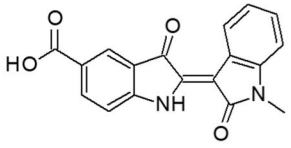
Indirubin-5'-carboxylic acid
Yield **27** %
Titer **162** mg L^{-1}



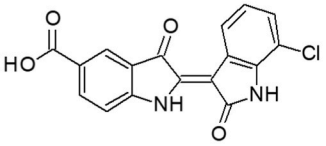
6-chloroindirubin-5'-carboxylic acid
Yield **30** %
Titer **167** mg L^{-1}



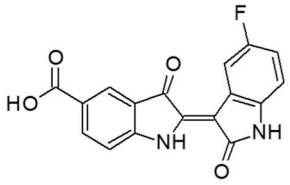
6-bromoindirubin-5'-carboxylic acid
Yield **26** %
Titer **185** mg L^{-1}



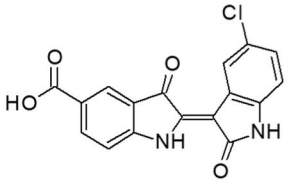
1-methylindirubin-5'-carboxylic acid
Yield **30** %
Titer **182** mg L^{-1}



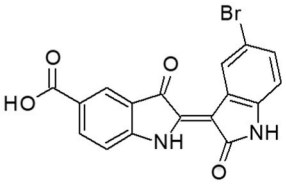
7-chloroindirubin-5'-carboxylic acid
Yield **46** %
Titer **164** mg L^{-1}



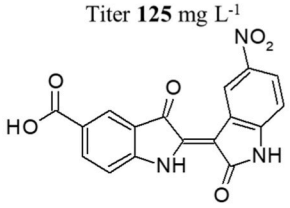
5-fluoroindirubin-5'-carboxylic acid
Yield **14** %
Titer **125** mg L^{-1}



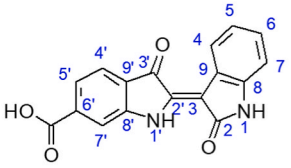
5-chloroindirubin-5'-carboxylic acid
Yield **24** %
Titer **154** mg L^{-1}



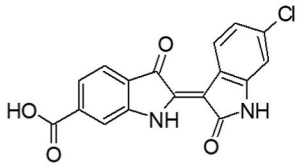
5-bromoindirubin-5'-carboxylic acid
Yield **30** %
Titer **193** mg L^{-1}



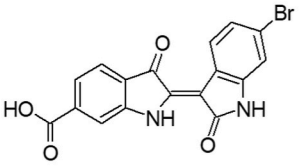
5-nitroindirubin-5'-carboxylic acid
Yield **36** %
Titer **124** mg L^{-1}



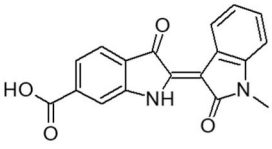
Indirubin-6'-carboxylic acid
Yield **41** %
Titer **233** mg L^{-1}



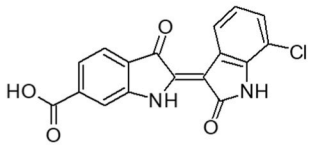
6-chloroindirubin-6'-carboxylic acid
Yield **40** %
Titer **280** mg L^{-1}



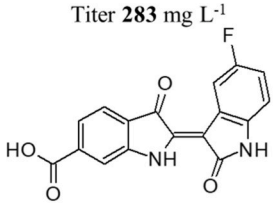
6-bromoindirubin-6'-carboxylic acid
Yield **42** %
Titer **283** mg L^{-1}



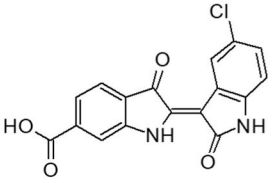
1-methylindirubin-6'-carboxylic acid
Yield **43** %
Titer **273** mg L^{-1}



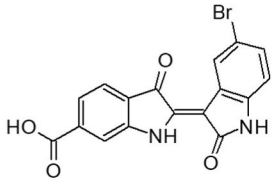
7-chloroindirubin-6'-carboxylic acid
Yield **36** %
Titer **290** mg L^{-1}



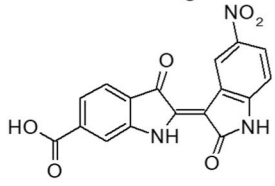
5-fluoroindirubin-6'-carboxylic acid
Yield **41** %
Titer **214** mg L^{-1}



5-chloroindirubin-6'-carboxylic acid
Yield **61** %
Titer **262** mg L^{-1}



5-bromoindirubin-6'-carboxylic acid
Yield **42** %
Titer **327** mg L^{-1}



5-nitroindirubin-6'-carboxylic acid
Yield **56** %
Titer **231** mg L^{-1}

Fig. 5. Structures of the synthesized monocarboxyindirubins under the optimized bioconversion conditions (yields and titers are given).

Table 1
Properties of the purified indirubin carboxylic acids. Absorption maxima were determined in H_2O, unless stated otherwise.

Compound	λ_{max}, nm	Solubility in H_2O, mM	Reference
Indirubin	546 (DMSO)	0.048	[31]
Indirubin-7,7′-dicarboxylic acid	548	13	[21]
Indirubin-5′-carboxylic acid	542	5.3	This work
Indirubin-6′-carboxylic acid	557	5.6	This work
1-methylindirubin-5′-carboxylic acid	540	13	This work
1-methylindirubin-6′-carboxylic acid	553	6.4	This work
5-chloroindirubin-5′-carboxylic acid	528	61.8	This work
5-chloroindirubin-6′-carboxylic acid	554	33.6	This work
6-chloroindirubin-5′-carboxylic acid	543	39.2	This work
6-chloroindirubin-6′-carboxylic acid	555	15.5	This work
7-chloroindirubin-5′-carboxylic acid	534	41.1	This work
7-chloroindirubin-6′-carboxylic acid	552	49	This work
5-bromoindirubin-5′-carboxylic acid	539	73	This work
5-bromoindirubin-6′-carboxylic acid	555	65	This work
6-bromoindirubin-5′-carboxylic acid	544	42.8	This work
6-bromoindirubin-6′-carboxylic acid	556	43.3	This work
5-fluoroindirubin-5′-carboxylic acid	534	18.16	This work
5-fluoroindirubin-6′-carboxylic acid	553	23.1	This work
5-nitroindirubin-5′-carboxylic acid	535	78.3	This work
5-nitroindirubin-6′-carboxylic acid	547	46.8	This work

amounts in *E. coli* compared to the production in *P. putida* (Fig. 3A). Low production of 5-fluoroindirubin-5′-carboxylic acid and 5-nitroindirubin-5′-carboxylic acid was also observed under all tested conditions, leaving the regular conditions (conditions **B**) in *P. putida* KT2440 as the preferred method for the synthesis of these compounds with the lowest level of by-products.

It has been reported that the addition of higher concentrations of 2-indolinone increases the production of indirubin [19]. However, the tested different molar ratios of indole carboxylic acid:2-indolinone derivative did not have a pronounced effect on the ratio of products in the bioconversions with *P. putida* KT2440 cells. Only in some cases, the yield of indirubin derivative increased with a higher concentration of 2-indolinone. For example, the yield of 5-chloroindirubin-5′-carboxylic acid increased by 33% with a 1:2 M ratio under the optimized conditions with *P. putida* KT2440 cells (Fig. 3C). On the other hand, the yield of most of the indirubin-5′-carboxylic acid derivatives was similar at different concentrations of 2-indolinone derivative or even lower at a higher concentration of 2-indolinone derivative, which was the case for the synthesis of 5-bromoindirubin-5′-carboxylic acid. Thus, a 1:1 M ratio of indole-5-carboxylic acid:2-indolinone derivative was chosen for the synthesis of most of the indirubin-5′-carboxylic acid derivatives as a cheaper and less stressful method for the bacterial cells.

Transferring the synthesis of indirubin-6′-carboxylic acid derivatives with Hind8 to *P. putida* KT2440 from *E. coli* cells resulted in a 1.5–5-fold increase in productivity and low levels of by-products under the optimized conditions (Fig. 4). In the case of 1-methylindirubin-6′-carboxylic acid, the indirubin: indigo ratio shifted dramatically from 1:10 to 14:1. Similar to 5-nitroindirubin-5′-carboxylic acid, the most efficient synthesis of 5-nitroindirubin-6′-carboxylic acid occurred in *P. putida* KT2440 under regular conditions. These bioconversion results suggest that *P. putida* KT2440 is a superior host for the 2-indolinone-based synthesis of monocarboxyindirubins, compared to *E. coli* HMS174 Δ*tnaA*.

Similar to the synthesis of indirubin-5′-carboxylic acid derivatives, varying concentrations of 2-indolinone derivatives proved to have even less effect on the yield of indirubin-6′-carboxylic acid derivatives. This can be explained by the very high yields (>80%) of indirubin-6′-carboxylic acid derivatives even when equimolar ratios of the substrates were used under optimized conditions with *P. putida* KT2440 cells (Fig. 4C).

3.3. Properties of synthesized indigoids

After determining the optimal conditions for the synthesis of monocarboxyindirubins, a total of 18 compounds were produced and subsequently purified by flash chromatography, and the purity was confirmed by HPLC/MS (Figs. S2–S19). The final yield of monocarboxyindirubins ranged from 14 to 61% (Fig. 5). Approximately 5–15 mg of each purified compound was subjected to NMR analysis, which confirmed the structures of monocarboxyindirubins (see Supplementary Material). Optical absorption spectra of the purified indirubins showed absorption maxima at 528–544 nm for derivatives of indirubin-5′-carboxylic acid and maxima at 547–557 nm for derivatives of indirubin-6′-carboxylic acid (Table 1).

We have previously shown that dicarboxyindigo and dicarboxyindirubin have an increased solubility in water, compared to non-carboxylated indigoids [21]. Furthermore, increased solubility in water correlates with the antiproliferative activity of indirubins [6,8]. Therefore, we investigated the water-solubility of the purified monocarboxyindirubins. It was found that all synthesized monocarboxyindirubins were substantially more soluble in water than unsubstituted indirubin. Furthermore, some of the side chains introduced into the indirubin molecule increased the solubility in water even more. For instance, nitro- and bromine-containing monocarboxyindirubins and most of the chlorine-containing monocarboxyindirubins showed more than a 1000-fold increase in water solubility, compared to indirubin (Table 1).

3.4. Antiproliferative activity of water-soluble asymmetric indirubin carboxylic acids

Indirubin is known to have anticancer activity and there is evidence that this is dependent on the water solubility of the indirubin tested [7]. Since the monocarboxyindirubin derivatives described in this work showed high solubility in water, we decided to evaluate the antiproliferative activity of these compounds. Two different cell lines were used for this experiment: HCT116 cells (human colon cancer cells) and MCF7 cells (human epithelial cells isolated from the breast tissue of an adult female, patient with metastatic adenocarcinoma). Cells were exposed to different concentrations of the purified monocarboxyindirubins for 48 h and the viability was measured by the MTT assay. All compounds tested showed statistically significant antiproliferative activity. While 1-methylindirubin-5′-carboxylic acid displayed the highest antiproliferative activity for both cell lines (Fig. 6), 5-bromoindirubin-5′-carboxylic acid and 5-nitroindirubin-6′-carboxylic acid showed high antiproliferative effects for the HCT116 cell line only. Interestingly, substrates for the synthesis of monocarboxyindirubins (indole-5-carboxylic acid, indole-6-carboxylic acid, and 2-indolinone) all exhibited potent activity against the MCF7 cell line, but not against HCT116 cells. Taken together, these data indicate that different cell lines may be differently affected by water-soluble indirubins and that certain monocarboxyindirubins, namely 1-methylindirubin-5′-carboxylic acid, represent a suitable lead compound for the development of monocarboxyindirubin-based anticancer agents.

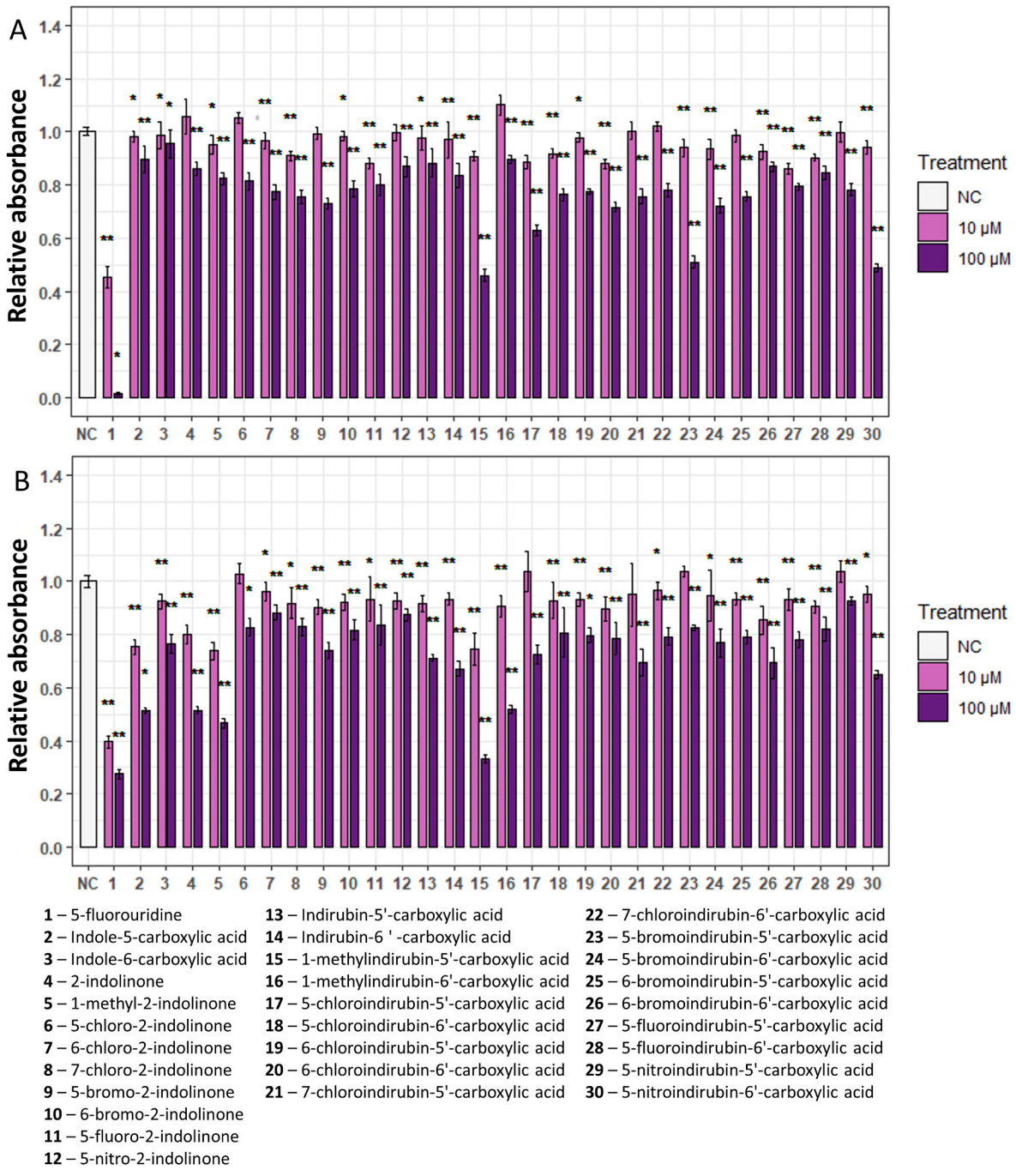


Fig. 6. Antiproliferative activity of 2-indolinone and indirubin carboxylic acid derivatives against different cell lines: A – HCT116 cell line, B – MCF7 cell line. Both cell lines were treated with 10 μM and 100 μM of different indirubin carboxylic acids for 48 h. 5-fluorouridine was used as a positive control. Statistical significance is indicated by *p*-values, where the symbol * designates $p < 0.05$, whereas the symbol ** designates $p < 0.01$ for untreated cells – negative control (NC).

4. Discussion

To the best of our knowledge, none of the 18 indirubin derivatives reported herein have been synthesized enzymatically so far. In total, 4 of those 18 indirubin compounds have been chemically synthesized: indirubin-5′-carboxylic acid [9–11], indirubin-6′-carboxylic acid [10], 5-fluoroindirubin-5′-carboxylic acid [12] and 6-bromoindirubin-6′-carboxylic acid [32]. These compounds are also known to have antiproliferative effects. While no biological activity has been reported on 5-fluoroindirubin-5′-carboxylic acid and 6-bromoindirubin-6′-carboxylic acid, indirubin-6′-carboxylic acid was found to specifically inhibit casein kinase 1 and kinases DYRK1a and DYRK2, but not kinases CDK5 and GSK3 [10]. In our work, indirubin-6′-carboxylic acid did not show high antiproliferative activity against two different cell lines. A reasonable amount of biological data is available on indirubin-5′-carboxylic acid and its derivatives. It was found that indirubin-5′-carboxylic acid is a non-specific inhibitor of several kinases (CDK5, GSK3, CK1, DYRK1a, and DYRK2) [10]. On the other hand, indirubin-5′-carboxylic acid does not inhibit the growth of the cell lines LXFL529L (human large-cell lung cancer) and MCF7 (human breast adenocarcinoma) [9], which is consistent with our results. These data suggest that activity testing against the target cell components can help to discover bioactive compounds, that might otherwise be missed, and vice versa – inhibition of one or more cellular components does not

necessarily translate phenotypically into the inhibition of proliferation.

Among the 18 indirubins tested in this work, 1-methylindirubin-5′-carboxylic acid showed the highest antiproliferative activity against both cell lines. Interestingly, indirubin compounds with the same scaffold have been reported in the literature with inhibitory effects. For example, 7-bromo-1-methyl-5′-indirubin-3′-oxime, the closest reported compound to 1-methylindirubin-5′-carboxylic acid, is a potent selective inhibitor of CDK5, DYRK1a, and DYRK2 kinases [10]. Removal of substituent groups (3′-oxime, N1-methyl, and 7-bromine) altered the selectivity against different kinases, but the overall inhibitory effect remained. Furthermore, the removal of the 5′-carboxyl or conversion to the methyl ester completely abolished the activity [10]. Taken together, it appears that indirubin-5′-carboxylic acid represents a scaffold for a potent kinase inhibitor, and the decoration of this scaffold with different substituent groups allows for modulation of the activity against different target kinases.

The method presented herein for the synthesis of asymmetric indirubins offers several advantages over the existing enzymatic methods. In general, the addition of 2-indolinone or its derivatives to the reaction mixture of enzymatic indole oxidation allows the production of indirubin derivatives with the formation of by-products as well. The number of by-products formed depends on the activity of the enzyme. For example, a flavin-containing monooxygenase from *Methylophaga aminisulfidivorans* produces a mixture of 3-hydroxyindole and 2-hydroxyindole during indole oxidation, thus forming indirubin naturally [18]. The addition of 2-indolinone into such a reaction mixture shifts the yield toward indirubin, but the addition of the 2-indolinone derivative produces a corresponding indirubin derivative, resulting in three reaction products: indigo, indirubin, and indirubin derivative. In contrast, Hind8 and PML G109Q enzymes not only possess the unique property of oxidizing indole carboxylic acids, but also appear to do it specifically at the C-3 position as no formation of other indigoids was observed [21,25], except for indole-7-carboxylic acid [21]. Furthermore, the obtained titers (up to 327 mg L^{-1}) and yields (up to 60%) of monocarboxyindirubins were comparable to the continuous fermentation system where 2-indolinone was added to redirect the oxidation of indole towards indirubin [20]. The combination of fewer reaction products, high production titers, and a high indirubin: indigo ratio allows for easier purification and higher recovery of the target compounds. Finally, it should be possible to use any 2-indolinone derivative and obtain the corresponding indirubin derivative since the 2-indolinone is not directly involved in the enzymatic reaction.

Additionally, the biosynthetic method for preparation of monocarboxyindirubins described herein can be compared to chemical procedures. As the chemical synthesis of carboxyindirubins usually consists of several steps (preparation of the indoxyl acetate derivatives, condensation with isatin under acidic conditions [9,10]), the presented bioconversion is a single reaction occurring under mild conditions. On the other hand, while the yields in chemical synthesis reach 80–90%, the biocatalytic synthesis of monocarboxyindirubins can result into a mixture of products thus requiring an extensive purification step. In the case of the biocatalytic synthesis of monocarboxyindirubins by the Hind8 and PML monooxygenases, indigo dicarboxylic acid is formed as a side product, which was minimized by optimizing the bioconversion conditions and shifting the reaction yield towards indirubin.

5. Conclusions

Overall, our method expands the variety of asymmetric indirubins that can be synthesized enzymatically. It employs indole carboxylic acids and 2-indolinone derivatives as substrates and bacterial oxygenases Hind8 or PML G109Q as catalysts for the *in vivo* reactions. Furthermore, high product titers (up to 327 mg L^{-1}) and satisfactory yields (up to 60%) were achieved by transferring the bioconversion platform into the *P. putida* KT2440 host. The synthesized monocarboxyindirubins retained the characteristic feature of high water-solubility and showed potential as antiproliferative agents.

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CRediT authorship contribution statement

Mikas Sadauskas: Conceptualization, Methodology, Investigation, Visualization, Writing – original draft. **Martynas Jakutis:** Investigation. **Vytautas Petkevičius:** Methodology, Investigation, Writing – review & editing. **Martynas Malikėnas:** Methodology, Investigation, Writing – review & editing. **Viktorija Preitakaitė:** Methodology, Investigation, Visualization, Writing – review & editing. **Justas Vaitekūnas:** Methodology, Investigation, Writing – review & editing. **Rolandas Meškys:** Conceptualization, Methodology, Resources, Writing – review & editing, Supervision, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.dyepig.2023.111585.

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